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"A DNA Damage Response (DDR) –independent Role for the Ataxia-Telangiectasia Mutated (ATM) Gene Product"

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“A DNA Damage Response (DDR) – independent Role for the Ataxia-Telangiectasia Mutated (ATM) Gene Product”

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- **D'Angiolella V, Palazzo L, Santarpia C, Costanzo V, Grieco D**. Role for non-proteolytic control of M-phase-promoting factor activity at M-phase exit. PLoS One 2007; 2(2): e247.

- **Visconti R, Palazzo L, Grieco D.** Requirement for proteolysis in spindle assembly checkpoint silencing. Cell Cycle 2010; 9(3): 564-9.

- **Visconti R, Palazzo L, Grieco D.** Role for the RNA Polymerase II-CTD phosphatase Fcp1 in cyclin B-dependent kinase 1 inactivation at mitotis exit. In preparation.

- **Palazzo L, Visconti R., Della Monica R., Grieco D.** ATM is required for the Tankyrase-1-dependent Poly(ADP-ribosyl)ation of Nuclear Mitotic Apparatus Protein 1 (NuMA1). In preparation.

2. ABSTRACT

Ataxia-Telangiectasia (A-T) is a recessive hereditary syndrome characterized by cerebellar degeneration, telangiectasia, precocious aging, immunodeficiency, cancer predisposition and insulin-resistant diabetes. A-T is caused by defects in Ataxia-Telagiectasia Mutated (*Atm*) gene. *Atm* encodes a ser/thr kinase (ATM) of the PI3 kinase family that plays a crucial role in the DNA damage response (DDR). Although some A-T features are easily explained by defects in DDR, others, like precocious aging, insulin-resistant diabetes and the recently described whole chromosome instability, are not. We, thus, searched for possible DDR-independent roles of ATM. To this end, we analyzed human cells in which ATM was chemically or genetically downregulated in the absence of any DNA damaging insult. We observed that ATM downregulation induced abnormal mitotic figures. The phenotypes ranged from abnormal mitotic spindles that were displaced from the cell centre, indicating loss of cortical interaction of astral microtubules, to spindles with extra poles that slowly coalesced to form pseudo-bipolar spindles. Often the extra poles lacked the centrosome marker gamma-tubulin. These alterations resembled that of previously described mitotic phenotypes induced by genetic knock-down of the poly(ADP-ribosyl)-polymerase Tankyrase 1 (TNKS1). The mitotic phenotypes have been ascribed to reduced TNKS1-dependent poly(ADP-ribosyl)ation of the Nuclear and Mitotic Apparatus protein 1 (NuMA1). Indeed, we found that ATM down-regulation impaired mitotic poly(ADP-ribosyl)ation of NuMA1. We further observed that ATM physically interacted with TNKS1. ATM activity was required for TNKS1-dependent poly(ADP-ribosyl)ation of NuMa1, perhaps through ATM-dependent phosphorylation of NuMa1, but not for ATM-TNKS1 interaction. Previous observations have shown that TNKS1 controls telomere length by (poly(ADP-ribosyl)ating TRF1 and glucose uptake by regulating insulin-dependent transport into the plasma-membrane of the glucose transporter Glut-4. We propose that the ATM-dependent control of TNKS1 function, independently of DDR, could explain several A-T features and shed light on new therapeutic approaches for the A-T syndrome.

3. THESYS MAIN BODY

3.1 BACKGROUND

3.1.1 ATAXIA-TELANGIECTASIA (A-T)

Ataxia-telangiectasia (A-T), first described by Boder and Sedgwick (1957), is an autosomal recessive human hereditary syndrome. It is a multisystem disease characterized by progressive cerebellar ataxia, oculocutaneous telangiectasia (dilated blood vessels) (Boder and Sedgwick 1957; Boder 1985), radiosensitivity (Good et al. 1964), hypogonadism (Boder 1985), cancer predisposition, in particular lymphoid malignancies (Good 1972; Boder 1975) and breast cancer (Walsh and King 2007; Campeau et al. 2008), cellular and humoral immunodeficiency (Waldmann 1982;), insulin-resistant diabetes mellitus (Schalch et al. 1970**)** and precocious aging (Good 1972) (Fig. 1).

Ataxia is the presenting feature of A-T syndrome and becomes manifested when child begins to walk. The main cause of ataxia in A-T patients is cerebellar degeneration involving Purkinje, granular cells and basket cells. Moreover, changes in the cerebrum are also found (Boder 1985).

Dilated blood vessels (telangiectasia) and immunodeficiency become apparent after the onset of ataxia, between 2 and 8 years of age. However, telangiectasia and defects in immune system do not develop in all A-T patients with the same morbidity (Boder 1985; McFarlin et al. 1972).

The immunodeficiency phenotype in A-T patients is variable. Patients with A-T may have defects in both T-lymphocyte (cellular arm) and B-lymphocyte (humoral arm) systems (Boder E. 1985; McFarlin et al. 1972). Abnormalities in the cellular-arm of the immune system are usually associated with a small or immature thymus gland. Thus, A-T patients also may have reduced numbers of circulating T-lymphocytes. However, the low number of T-lymphocytes generally does not increase the patient's susceptibility to infection (Nowak-Wegrzyn et al. 2004). The immunological deficiency concerning humoral-arm of immunity is frequent (60-70% of A-T patients) and consists in deficiency or absence of IgA, in frequent absence of IgE (Nowak-Wegrzyn et al. 2004; McKinnon 2004). A-T patients with defects in only one branch of immune system generally have infective respiratory diseases that are not life-threatening (McFarlin et al. 1972; Nowak-Wegrzyn et al. 2004).

Finally, patients with A-T have an increased risk for developing cancer, particularly lymphoid malignancies, like lymphoma and leukemia (Good 1972; Boder 1975), and susceptibility for breast cancer, found also in A-T carriers (Walsh and King 2007; Campeau et al. 2008).

The cause of death in A-T patients is ultimately linked to bronchopulmonary disease (pneumonia or chronic lung disease), cerebellar degeneration and cancers (Lavin and Shiloh, 1997; Lefton-Greif et al. 2000; Nowak-Wegrzyn et al. 2004; McKinnon 2004).

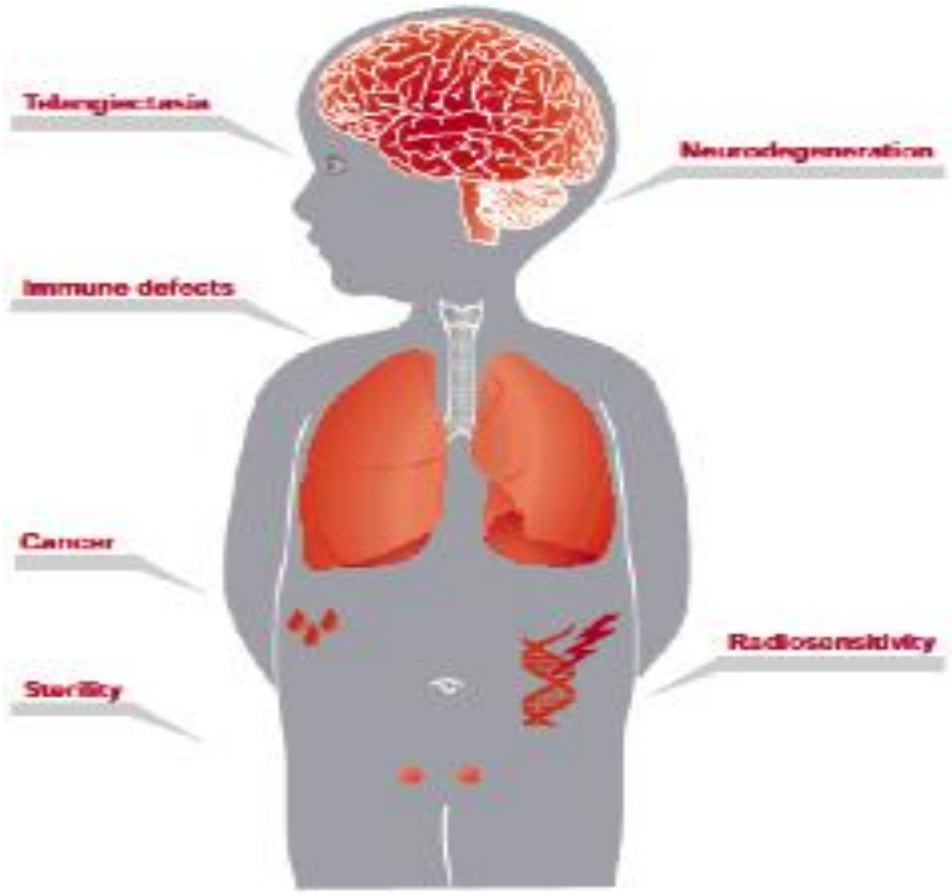

Fig1. Schematic representation of the A-T syndrome features. From McKinnon, 2004. Ataxia-Telangiectasia is a hereditary syndrome characterized by cerebellar neurodegeneration, ocular telangiectasia, immune system defects, radiosensitivity, cancer predisposition and gonadal atrophy.

3.1.2 **ATM functions in the DNA Damage Response (DDR)**

The gene mutated in A-T syndrome**,** "Ataxia-Telangiectasia Mutated" (*Atm*) gene, was localized to chromosome 11q22-23 (Gatti et al. 1988) and cloned by positional cloning (Savitsky et al. 1995). *Atm* gene encodes for a large Ser/Thr protein kinase, approximately 350 kDa (ATM). Several types of mutations have been indentified in the *Atm* gene, most being truncating or splice-site mutations. Some *Atm* alleles lead to an unstable protein, other to a reduced amount of functional protein or normal amounts with dramatic reduction of enzymatic activity. The types of mutation may explain different severity of A-T syndrome, although neurodegeneration is always present (Stewart et al. 2001, McKinnon 2004).

The ATM protein belongs to phosphatidylinositol-3OH- kinase related protein kinase (PIKK) family (Savitsky et al. 1995). All members of the PIKK family are large Ser/Thr protein kinases involved in signaling following cellular stress. The ATM consensus phosphorylation motif is Ser/Thr followed by Glutamine (Kim et al. 1999). PIKK family includes ATR (ATM and Rad3 related protein kinase), DNA-PKcs (DNA dependent protein kinase catalytic subunit), mTOR (mammalian target of rapamycin) and hSMG1. All PIKK members share common domain including N-terminal HEAT domain repeats, a FAT domain, a kinase domain and a C-Terminal FAT-C domain (Lovejoy and Cortez 2009). (Fig2)

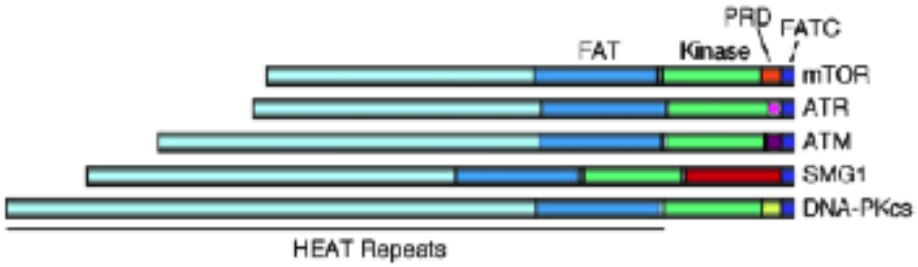

Fig2. PIKK family members. From Lovejoy and Cortez 2009. The PIKK family members have a C-Terminal protein kinase domain flanked on either side by an N-Terminal FAT domain and a C-Terminal FAT-C domain. N-Termini are composed of HEAT repeats.

ATM has a central role in DNA Damage Response (DDR), being ATM in particular activated by DNA double strand breaks (DSBs). ATM is recruited to DSBs indirectly through binding Mre11-Rad50-Nbs1 (MRN) complex (Lavin 2007). Also DNA-PKcs is activated by DSBs and it is recruited to DSBs by interacting with the end binding heterodimer Ku70/80 (Smith and Jackson 1999). Binding of Ku70/80 to DNA ends provides a scaffold for the association of DNA-PKcs and other proteins involved Non-Homologous-End-Joining (NHER), whereas ATM is involved in DDR resolution mediated by Homologous Recombination (Lovejoy and Cortez 2009; Derheimer and Kastan 2010). ATR is, instead, recruited to single-strand DNA (ssDNA) through its binding partner ATRIP, which indirectly recognizes ssDNA through an interaction with the ssDNA binding protein replication protein A (RPA) (Cimprich and Cortez 2008; Lovejoy and Cortez 2009). ATM protein kinase can be activated by DSBs caused by exposure to ionizing radiations, by DSBs as a consequence of T-Cell and B-Cell receptor gene rearrangement or by DSBs caused by inhibition of Topoisomerase.
Whether ATM must be recruited to DSBs to be activated is less clear.
DSBs induce ATM autophosphorylation on Ser1981 and monomer formation (Bakkenist and Kastan 2003**).** Autophosphorylation at ser1981 is considered a sign of ATM activation, including localization to DSBs and activation of ATM kinase activity. However, in vitro studies using recombinant proteins showed that ATM S1981A mutant binds DNA ends and has kinase activity (Lee and Paull 2005). By contrast, in vivo studies have shown that ATM localization and stabilization to DSBs in human requires autophosphorylation (So et al.; 2007), but ATM knock-out mice complemented with a non-phosphorylatable ATM version at S1987 (mouse homologue of human ATM serine 1981) had normal ATM-dependent phosphorylation of ATM substrates after DNA damage and localization of ATM to DSBs (Pellegrini et al 2006). This data may suggest that ATM autophosphorylation could be necessary for localization to DSBs but could be not essential for other putative DDR-independent functions that require ATM kinase activity. Recently, it has been demonstrated by Guo et al. that ATM was activated by oxidative stress, ATM was phosphorylated in S1981 following challenge with H2O2**.** However autophosphorylation of ATM appeared not to be essential for H_2O_2-mediated activation of ATM, because full activity was observed with the $Ser^{1981} \rightarrow Ala^{1981}$ (S1981A) autophosphorylation site mutant, indicating that oxidative environment may induce conformational changes in ATM structure able to activate ATM in absence of DNA DSBs. **(**Guo et al., 2010**)**.
Following DSBs-dependent activation, ATM is also required to stop cell cycle progression. For instance, ATM efficiently stabilizes the tumor suppressor p53, thus playing a critical role in the G1/S checkpoint (Kastan et al 1991). ATM is able to phosphorylate not only p53 but also MDM2 and Chk2, proteins that interact with p53. The ATM-dependent induction of p53 allows the transcription of p53-target genes, in particular the cyclin-dependent kinase (CDK) inhibitor p21. Induction of p21 gene transcription results in a cell cycle arrest in G1/S phase (Kastan and Lim 2000).

In contrast, the intra S-phase arrest after IR exposure requires ATM, but not p53. The first ATM target in the radiation-induced intra S-phase checkpoint is p95/NBS1 (Lim et al. 2000). ATM mediated S-phase arrest is also BRCA1 (Breast Cancer Associated 1)-dependent. ATM is able to phosphorylate BRCA1 on multiple sites, and these different phosphorylation events elicit different effects on cell cycle progression. Phosphorylation on ser1387-BRCA1 is necessary for S-phase arrest following ionizing radiation (Xu et al 2002), while phosphorylation on ser1423-BRCA1 is necessary for the ATM-dependent G2/M arrest (Xu et al 2001). ATM- mediated phosphorylation of SMC1 and FANCD2 are also shown to be important for IR induced S-phase arrest (Taniguchi et al. 2002; Kitagawa et al. 2004). Finally, ATM phosphorylates and activates Chk2 kinase on Thr68 leading to Chk2-dependent phosphorylation and inhibition of Cdc25A. Cdc25A is the phosphatase able to activate CycE/Cdk2 complex. Chk2-dependent inhibition of Cdc25A results in arrest in S-phase. The Chk2- mediated inhibition of Cdc25 family of phosphatases is also important for the activation of G2/M arrest, inhibiting CycA-CycB/Cdk1 complex (Lavin 2008). (Fig3)

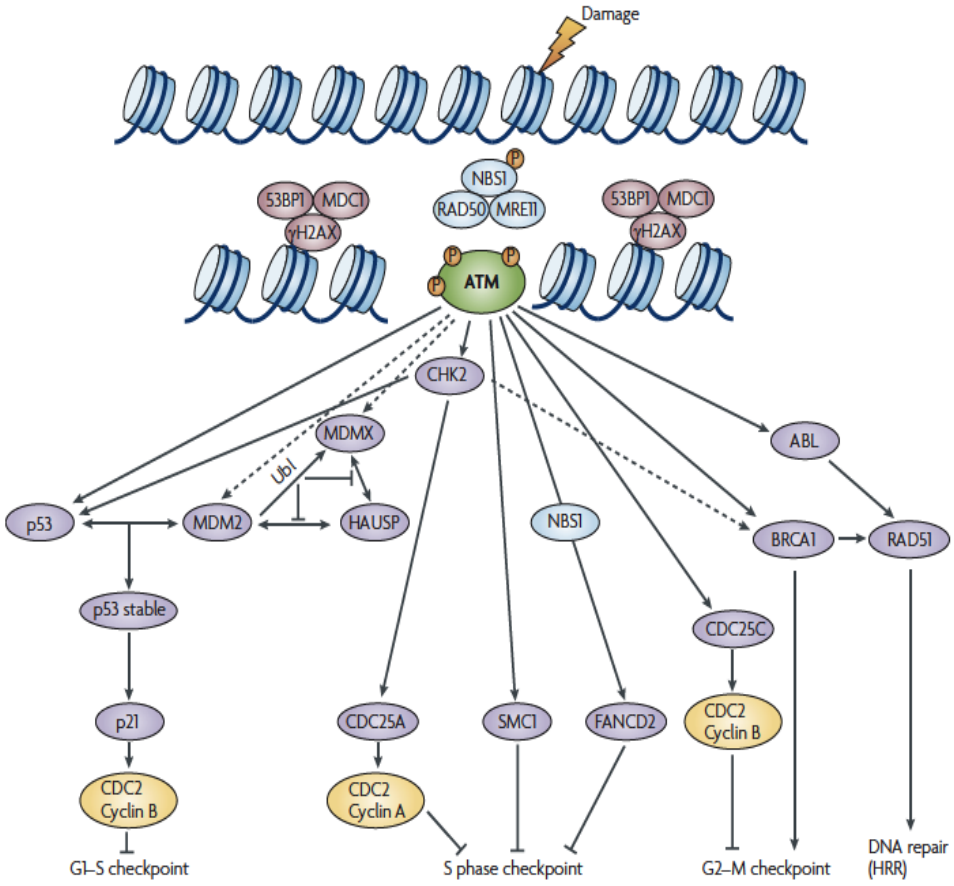

Fig3. DNA damage-dependent functions of ATM. From Lavin 2008. ATM protein has a central role in DNA damage repair. DNA DSBs are recognized by the MRN complex. The MRN complex recruits and activates ATM. Monomeric and autophosphorylated ATM provides to stop the cell cycle in a p53 -dependent (G1/S checkpoint) or –independent manner (intra S checkpoint and G2/M checkpoint). Moreover, DNA damage activated ATM activates and regulates the Homologous Recombination Repair (HRR) pathway.

ATM does not only block the cell cycle in presence of DDR but it is also directly involved in the mechanisms of DNA damage repair. It has in fact been shown that localization and activation of ATM on the DBSs is MRN (Mre11-Rad50-Nbs1)-dependent. MRN complex is the sensor of DNA damage. MRN complex is rapidly recruited to the DNA DSBs sites in ATM-independent manner (Lavin 2007). It was also reported that Mre11 generates small DNA fragments that can stimulate ATM kinase activation (Jazayeri et al. 2008).

Following MRN activation, ATM is recruited to the DSBs sites via interaction with Nbs1 (Falck J. et al 2005). Moreover, in order to promote a positive loop, MRN complex is a substrate for ATM (Kitagawa et al. 2004; Lee and Paull 2007). These findings show that MRN complex is necessary for full ATM activation and localization.

After localization to sites of DNA damage, ATM phosphorylates on Ser139 the histone variant H2AX to produce γH2AX (Burma et al 2001). A second substrate of ATM, MDC1, is recruited by γH2AX via its BRCT domain (Breast Cancer Susceptibility protein 1 C-terminal) and has a central role in the recognition and repair of DNA DSBs (Stucki and Jackson 2004). The adaptor protein MDC1 is, in turn, phosphorylated by ATM (Stucki and Jackson 2004). The formation of γH2AX and the recruitment of MDC1 on DNA damage foci provide a docking station for many components of the DNA damage repair, such as the ubiquitin ligases RNF8 and RNF168, P53BP, BRCA1, both of which are also phosphorylated by ATM, and the recombinase RAD51 (Lavin 2008) for the homologous recombination. (Fig. 4)

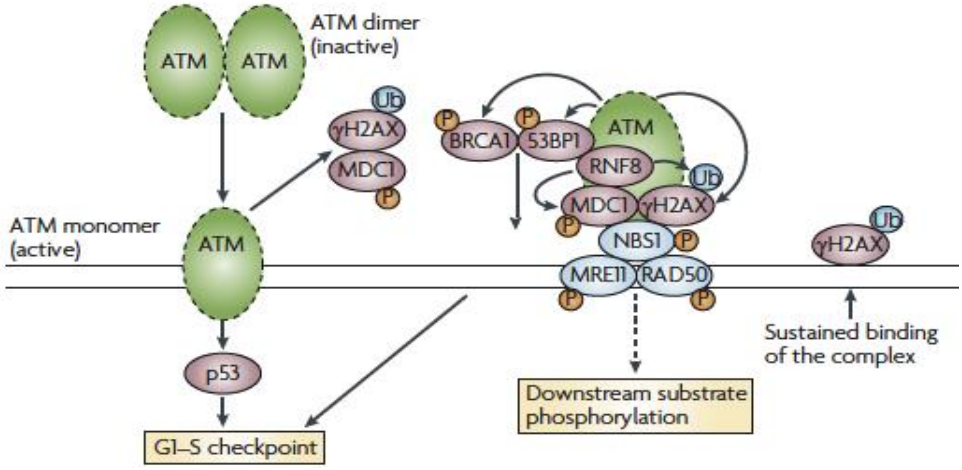

Fig4. ATM-dependent DNA damage repair cascade. From Lavin 2008. Monomeric and autophosphorylated ATM is recruited on DNA damage foci by the MRN complex. ATM-phosphorylated p53, arrests the cell cycle at the G1/S checkpoint. In order to localize and maintain DNA damage repair proteins on DNA damage foci, ATM phosphorylates H2AX and MDC1. The formation of γH2AX and the recruitment of MDC1 on DNA damage foci provide a docking station for ubiquitin ligases RNF8 and RNF168, P53BP, BRCA1 and the recombinase RAD51.

3.1.3 **Known ATM functions do not explain all the A-T features**

A-T syndrome is characterized by a variegated phenotype composed by cerebellar atrophy, oculomotor apraxia, telangiectasia, immunodeficiency, radiosensitivity, chromosomal instability, cancer predisposition, infertility, precocious aging and insulin resistance diabetes. Some of these features are easily explained by the known function of ATM protein kinase in DNA damage response. In fact, the immunodeficiency can be explained by the deficiency in homologous recombination repair system in A-T lymphocytes and in generation of non-functional T- or B- cell receptors. ATM is naturally activated by the physiological DNA breaks during the B and T cells maturation and differentiation as demonstrated in *Atm*-deficient mice (Lumsden et al. 2004) and in human derived lymphocytes (Bredemeyer et al. 2006). The ATM function in the safeguard of genome maintenance during lymphocytes maturation explains the lymphopenia and the increased predisposition to lymphoid malignancies with chromosomal translocation involving lymphocyte antigen receptor loci in Ataxia-telangiectasia (Bredemeyer et al. 2006).

DNA damage-dependent functions of ATM can also explain the radiosensitivity, the qualitative chromosomal instability (CIN) and the presence of chromosomal end-to-end fusions (Pandita et al 1995) found in A-T patients.

However, it is not clear how ATM can normally take part in cerebellar trophism, in the regulation of ocular veins size (Boder and Sedgwick 1957; Boder 1985), in the glucose response (Schalch et al. 1970), in the not-damaged telomeres maintenance (Wong et al. 2003).

3.1.4 **Mitosis, Telomeres length control and Glucose response: How is ATM involved in these processes?**

Thus, while immunodeficiency, radiosensitivity and cancer predisposition can be easily explained by the difficulty of A-T cells to repair DNA DSBs with homologous recombination, it is indeed difficult to understand how deficiency in the homologous recombination pathway may affect the insulin-response, the telomeres shortening and the neuronal viability.
In *Drosophila,* ATM homologue is essential for normal development of the nervous system. *Atm*-deficient mutants show extensive apoptosis in neuronal tissues and it was associated with frequent mitotic defects and chromosomal abnormalities (Silva et al. 2004). *Atm*-deficient mice display neurobehavioral deficits consistent with abnormal cerebellar function and, moreover, a dramatic increase of aneuploidy neurons in the frontal cortex (McConnell et al. 2004). These observations suggest that ATM loss of function is associated with neural genome instability, including aneuploidy, and contribute to neurodegeneration not only in $Atm^{-/-}$ mice but also in the human A-T brain. Recently, increased aneuploidization of the human cerebellum in A-T has been showed (Iourov et al. 2009). Therefore, the global aneuploidization of the brain is a newly uncovered genetic phenomenon in A-T syndrome. Moreover, evidence shows that ATM deficiency causes aneuplody both in vivo and in vitro, affecting tumorigenesis (Shen et al. 2005; Li et al. 2010).
Thus, ATM loss does not only lead genomic structural alterations such translocation, but also to numerical changes in whole chromosomes (aneuploidy). Aneuploidy is found in a large majority of tumors (Mertens et al. 1994), and this observation prompted Boveri to propose that cancer was caused by aneuploidy (Boveri 2008). It is well known that aneuploidy is caused by defects in chromosomes segregation during mitosis (Compton 2010). Aneuploidy arising through chromosome mis-segregation during meiosis is also a major cause of infertility and inherited defects (Hassold and Hunt 2001).
How ATM could affect chromosomes segregation is still largely unknown.
Another A-T feature not yet well understood is precocious aging. A major cause of ageing is the telomere erosion and loss of the stem cell compartments in the adult**.** Telomeres are a complex structure involving DNA, RNA, and proteins (shelterin complex) that protect the free end of chromosome form being recognized by DNA damage repair machinery as DSBs. The shelterin complex is composed by several proteins (TIN2, TRF1, TRF2, TPP1, POT1 and RAP) and it binds to the telomeric sequence and forms a t-loop structure, now know to be essential in the protection of telomeres and for their ability to elude the DNA damage sensing machinery (de Lange 2005; Palm and de Lange 2008). Among Shelterin components, TRF1 mediates telomere replication and TRF2 mediates the telomere protection (Broccoli et al. 1997; van Steensel and de Lange, 1997, van Steensel et al 1998). TRF1 is a negative regulator of telomere length by telomerase. TRF1 has to leave telomeres to allow telomerase to elongate them

after each round of chromosome replication. In order to dissociate form telomeres, TRF1 has to be Poly(ADP-ribosyl)ated by the PARP enzyme Tankyrase1 (Smith et al. 1998).
The most common observed defects affecting telomeres in humans are telomere end-to-end fusions and telomeres shortening, together these phenomena impairing cellular and whole organism viability (Metcalfe et al. 1996; Wong et al. 2003).
ATM has been shown to play a role in mammalian telomere length regulation. Intriguingly, the yeast homolog of *Atm*, *Tel1*, that plays only a minor role in the DNA damage response, is a key regulator of telomere function, controlling the recruitment of telomerase to the telomere. In fact, *Tel1* mutant strains show telomere hyper-recombination, telomere fusion, chromosome loss and progressive telomere shortening (Greenwell et al. 1995; DuBois et al. 2002). In mammalians, whereas it is well know the role of ATM when telomere shorten (Palm and de Lange 2008), it is still unclear how mammalian ATM plays any role in telomerase length maintenance. $Atm^{-/-}$ mice crossed with $mTR^{+/-}$ or $mTR^{-/-}$ mice (mouse telomerase RNA) show that mouse ATM plays an important role in telomere capping but it is dispensable for elongation of short telomeres by telomerase (Feldser et al.2006). Data obtained in mouse model do not exclude a role for ATM in human telomere length control. In fact, human and mouse telomeres show some differences in the control of access of the telomerase to the telomere, for example in the Tankyrase1-mediated Poly(ADP-rybosil)ation of the Shelterin subunit TRF1, necessary event for access of human telomerase to the telomeres. (Sbodio and Chi 2002)
Another intriguing feature of A-T is the predisposition to insulin resistance diabetes (Schlach et al. 1978). Bar et al. 1978 and Yang and Kastan 2000 shown that ATM was stimulated by insulin signaling and in some cell types ATM loss was led an adverse effect on insulin dependent signaling. Metabolic syndrome can be caused in part by insulin resistance, contributing to the development of atherosclerosis and obesity. The role of ATM in insulin signaling suggests the possibility that its dysfunction could contribute to the development of metabolic syndrome. In fact, loss of one or both alleles of *Atm* enhances the features of metabolic syndrome in *Apo* $E^{-/-}$ mice fed on a high-fat diet (Schneider et al. 2006). Chloroquine treatment decreases atherosclerosis and blood pressure and improved glucose tolerance in an ATM-dependent manner in *Apo* $E^{-/-}$ mice.
These data suggest a cytoplasmic role for ATM in this process. Indeed, ATM is localized not only in the nucleus but also in the cytoplasm, and in both peroxisomes and endosomes (Lim et al. 1998; Watters et al. 1999).

3.2 **AIMS OF THE STUDY**

Not all A-T features can easily be explained by loss of ATM functions in DNA damage repair. Our major research goal is to understand how ATM is able to influence cellular processes like chromosome segregation, precocious aging, telomeres attrition and insulin-dependent glucose transport. The aim of the present study is to contribute to a better understanding of the A-T syndrome pathogenesis.

Thus, the aims of this study are to:

1- Study the effects of ATM gene expression down-regulation and of ATM kinase activity inhibition in mammalian cells.
2- Analyze novel molecular pathways in which ATM may be involved.
3- Identify putative new ATM interacting proteins that may explain potential DDR-independent ATM functions.

3.3 MATERIALS AND METHODS

3.3.1 Cell culture methods

HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 1000 mg glucose/L, $NaHCO_3$ and pyridoxine-HCl. Human colon cancer Hct116 were grown in high glucose DMEM supplemented with Hygromycin B 100 μg/ml (Sigma Aldrich). Retinal Pigment Epithelium (RPE) hTERT-immortalized cells were a gift of Dr. Musacchio and were grown in DMEM/F12 (Sigma Aldrich). AHH1 human lymphoblastic EBV-transformed cells and GM03189 A-T human lymphoblastic EBV-transformed cells were a gift of Dr. Soddu and were grown in RPMI (Sigma Aldrich). All medium were supplemented with 10% Fetal Bovine Serum (Thermo Scientific HyClone), L-Glutamine and 1% Penicillin 10.000U/mL-Streptomycin 10mg/mL (Sigma Aldrich).
To induce mitotic arrest, cells were treated with Tymidine (Sigma Aldrich) 4 mM for 18 h, released by rinsing thoroughly with PBS and incubated in fresh medium. After 6 h incubation, cells were blocked again with Tymidine 4 mM for 14 h. Cells were washed thoroughly and incubated in fresh medium supplemented with Nocodazole (Calbiochem; 100 ng/mL for HeLa, Hct-116 and RPE; 1μg/, AHH1 and GM03189) for 13 h prior to harvest. For Nocodazole release, cells that detached form substrate were recovered by shake-off and washed once with phosphate-buffered saline (PBS) and once with fresh medium before incubation into fresh medium. For Immuno-fluorescence experiments, growing cells were plated onto cover-glasses and treated with the Cdk1-inhibitor RO-3306 10 μM (Calbiochem). Short-term treatment for up to 20 hrs results in fully reversible G2/M cell cycle arrest. Upon RO-3306 wash-out, cells were treated with MG-132 (Calbiochem) for 2-3 h, cover-glasses were harvested at different time point and processed for immuno-fluorescence. For the methaphase-arrest of AHH1 and GM03189, cells were first treated with thymidine for 10 hours and then Nocodazole-arrested. After 12 hours, cells were washed once with phosphate-buffered saline (PBS) and once with fresh medium before incubation into fresh medium containing MG-132 (Calbiochem) for 2-3 h and were spun onto microscope-slides at 1000 rpm for 3 min by cytospin centrifuge (Shandon). MG-132 was used at 40 μM and, when indicated, Ku55933 (Calbiochem) at 10 μM.

3.3.2 **Immuno-Fluorescence**

Cells grown on cover-glass and cells spun onto microscope-slides were fixed with 3.7% formaldehyde for 15 min at 4°C and permeabilized with 0.2% Triton-X-100 in PBS for 10 min. For γ-tubulin staining, cells were treated with ice-cold 100% methyl-alcohol at -20°C for 20 min. After blocking with 3% BSA in PBS for 1 hour, samples were incubated with primary antibodies in PBS + 1% BSA overnight at 4°C. After 3 PBS washes, samples were incubated with secondary antibodies (Jackson Immuno Research Laboratories) in PBS + 1% BSA for 1 hour at room temperature. DNA was stained by incubation for 10 min with 10 μg/mL Hoechst 33258 in PBS. Samples were observed using an Axiovert 200M inverted microscope equipped with the Apotome slider module (Zeiss) with a 68X objective.

3.3.3 **Cell-extract**

Cells were harvested and lysed in PBS 0.2% Nonidet P-40 50% v/v EB buffer (b-glycerolphoshate 80 mM, 15 mM $MgCl_2$, 20 mM EGTA pH 7.7), + NaF 10 mM (Sigma Aldrich), NaPP 2 mM (Sigma Aldrich), 5 μM of the PAR glycohydrolase inhibitor ADP-HPD (Calbiochem) and complete protease inhibitor cocktail tablet (Roche).
Lysates were incubated 30 min on ice and then spun two times for 20 min at 13,000 rpm in a refrigerated microfuge.
Laemmli sample buffer was added to aliquots of 25 μg (determined by Bio-Rad protein assay) of supernatant proteins. The samples were boiled for 10 min and fractionated by 6% (for proteins kDa > 100) SDS/PAGE and analysed by immunoblotting.

3.3.4 **Antibodies and Immuno-precipitation**

Aliquots of 1 - 1.5 mg of pre-cleared supernatant proteins were incubated with anti-pADPr (2 μg/mL; clone 10H, mouse monoclonal antibody, Tulip Biolabs), rabbit anti NuMa 1 (2 μg /mL; Novus Biological), rabbit anti-Tankyrase 1/2 [(H-350); 2 μg/mL; Santa Cruz Biotechnology], mouse anti-Flag 1 μg/mL (Sigma Aldrich) O.N. at 4°C. Antigen–antibody complexes were then bound to Protein G–Sepharose (Santa Cruz Biotechnology) at 4 °C with rocking for 1.5 h. Immunoprecipitation of ATM was performed with goat anti ATM agarose immobilized (2 μg/mL; Novus Biologicals) with rocking at 4°C O.N..
Immunoblots were incubated with following primary antibodies: rabbit anti-Tankyrase 1/2 [(H-350) 1:350; Santa Cruz Biotechnology], rabbit anti NuMA

1 [(H-300) 1:350; Santa Cruz Biotechnology], rabbit anti NuMa 1 (1:1500; Novus Biologicals), mouse anti ATM (1:1000; Novus Biologicals), rabbit anti PAR [poly(ADP-ribose)] (1:2000; BD Bioscience), pS(T)Q ATM/ATR substrates (1:1000; Cell Signaling), mouse anti Flag (1:1000; Sigma Anldrich), Rabbit anti TRF1 (1:1000, Santa Cruz Biotechnology) followed by horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG (Amersham; 1:2500). Bound antibody was detected using ECL lumi-light Western Blotting Substrate (Roche).

3.3.5 **DNA and siRNA transfection**

Flag-ATM plasmid was a generous gift of V. Costanzo. Tankyrase cDNA was dissected and cloned in M12 N-3XFlag by Genecopoeia. TRF1 ORF (Invitrogen) was cloned in Flag pCR 3.1.
Transfection was performed with FuGENE 6 Transfection Reagent (Roche) for 72 h.
HeLa cells were transfected without (mock) or with siRNA oligonucleotides pool (Dharmacon Research Inc.) directed against ATM using Dharmacon transfection reagent for 72 h, as described by the manufacturer.

3.4 **RESULTS AND DISCUSSION**

To gain further information on potential DDR-independent functions of ATM, we analysed mammalian cells that were either treated with an ATM kinase inhibitor (Ku-55933) or with small interfering RNAs (siRNA) to knock-down (KD) *Atm* gene expression in the absence of DNA damage stimuli. For ATM kinase inhibitor treatment, HeLa cells were grown on cover-slide and then incubated for 24-hours in the presence of 10 μM Ku-55933, a selective and commercially available ATM kinase inhibitor, or vehicle (DMSO). After, cells were analyzed by immunofluorescence (IF). Cells were, then, stained for the microtubule's subunit α-tubulin, the spindle pole marker NuMA1 and DNA. Relatively to the control cells, Ku-55933-treated cells showed peculiar mitotic abnormalities, like defects in chromosome alignment, chromosome scattering, supplementary spindle poles and spindle displacement from cell centre (Fig. 5). Indeed, Ku-55933-treated cells showed 45% of chromosome alignment defects and 25% of abnormal spindle microtubular structures, compared to 8% and 5% of chromosome alignment defects and spindle abnormalities, in control cells (Fig. 6A, 6B). Similar results (Fig. 7) were obtained by synchronizing cells at the G2 phase with the cdk1-inhibitor RO-3306, post DNA replication. After 20-hours of RO-3306 treatment, cells were washed and then treated with 40 μM of MG-132, a proteosome inhibitor, a treatment that allows cells to assemble mitotic spindles but arrests them in metaphase as cells cannot degrade mitotic cyclin B and inactivate the major cyclin-dependent kinase (cdk), cdk 1. Where indicated, cells were also treated with Ku-55933 or vehicle. After 2-hours treatment, cells were stained for α-tubulin, NuMA1 and DNA and analyzed by IF. The data (Fig. 7) demonstrated that similar mitotic abnormalities were obtained under these conditions, indicating that the mitotic alterations induced by ATM inhibition were not due to prior errors in DNA replication that might have occurred in the absence of ATM kinase activity.

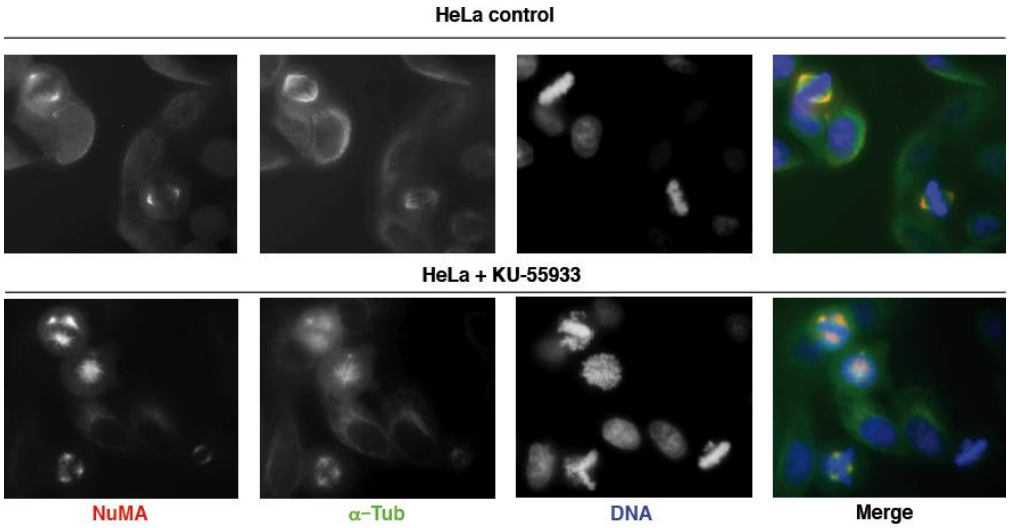

Fig5. ATM chemical inhibition results in defective chromosome alignment and mitotic spindle assembly. HeLa cells grown on cover-slide were treated with DMSO or 10 μM Ku-55933 and were stained after 24h for NuMA1, α-tubulin, and DNA. In the merge, NuMA1 staining is in red, α-tubulin in green and the DNA in blue. ATM inhibition led to assembly of extra NuMA1 positive spindle poles, indicating that spindle poles may be splitting or that new spindle poles may be assembling adjacent to existing ones.

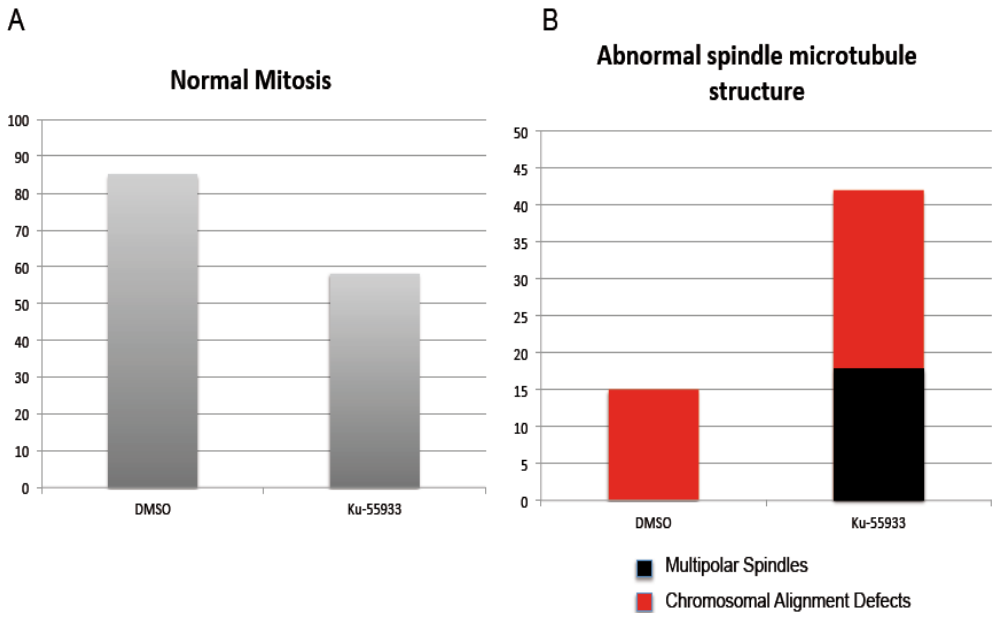

Fig 6. Quantification of the mitotic defects caused by ATM chemical inhibition. A Growing HeLa cells were treated with DMSO or 10 μM Ku-55933 for 24h. ATM chemical inhibiton led to 45 % of deranged mitosis with respect of 15% of control cells. **B** Ku-55933 treatment resulted in peculiar mitotic abnormalities, like defects in chromosome alignment, chromosome scattering, and spindle displacement from cell centre of mitosis in 45% of the mitosis. In the 15% of the abnormal mitosis were observed supplementary spindle poles.

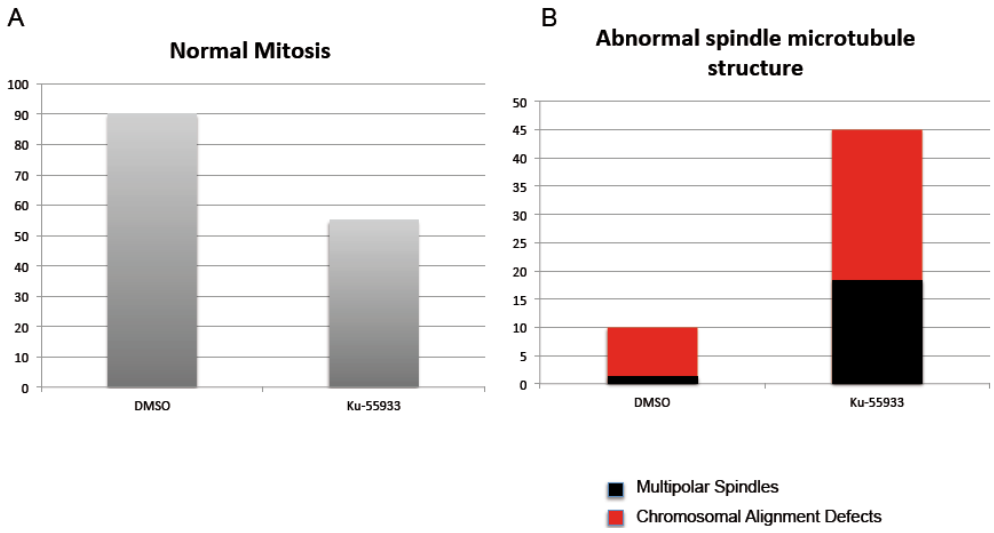

Fig 7. Quantification of the mitotic defects obtained by ATM chemical inhibition in RO-3306 synchronized cells. A and B HeLa cells were synchronized in G2/M with 9 μM RO-3306 and then treated with DMSO or 10 μM Ku-55933 in fresh medium containing 40 μM MG-132. ATM-inhibition led to 45% of abnormal mitosis. In 15% of abnormal mitosis were observed multipolar spindles.

To confirm with a different methodological approach that the phenotype obtained by Ku-55933 treatment was indeed caused by ATM down-regulation, we knocked-down ATM expression using a specific pool of four ATM gene targeting siRNAs. ATM siRNA eliminated 80-90 % of ATM protein at day 3 (Fig. 15B). ATM siRNAs-treated HeLa cells were grown onto cover-slides and analyzed by IF at day 3 after transfection, staining for □-tubulin, NuMA1 and DNA (Fig 8). ATM interfered cells showed 38% of deranged mitosis with 14% multipolar spindles. By contrast, control siRNAs-treated cells showed only 10% and 7% of chromosome alignment defects and aberrant mitotic figures, respectively (Fig9).

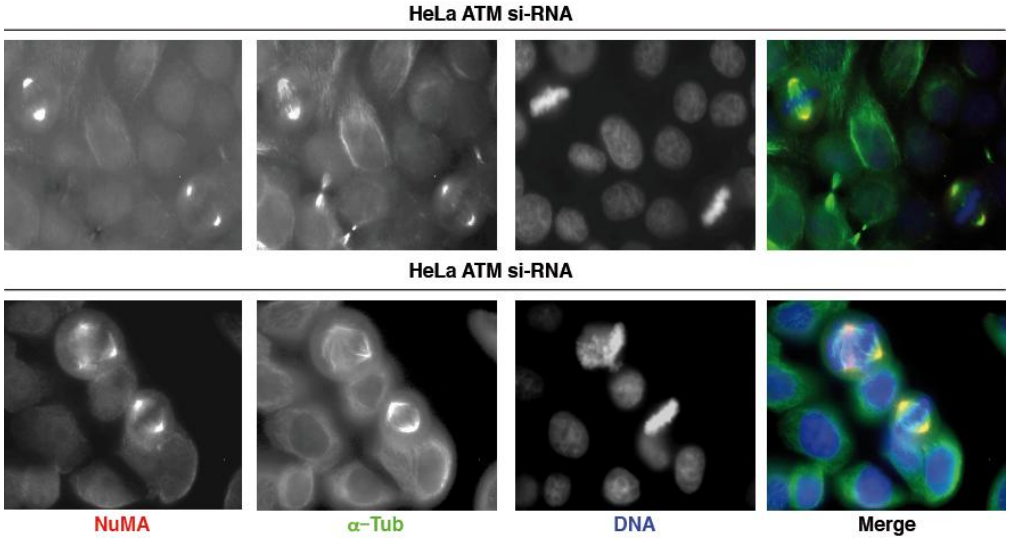

Fig 8. ATM is required for bipolar spindle assembly and chromosome alignment on metaphase plate. HeLa cells transfected with control small interfering RNA (siRNA) or ATM siRNA were grown on cover slides. At day 2 after transfection, cell were treated with 9 μM RO-3306 for 24-hours. At day 3, cells were released from RO-3306 and treated with 40 μM MG-132. Cells were then stained for NuMA1, α-tubulin and DNA. In the merge, NuMA1 staining is shown in red, α-tubulin in green and DNA in blue. ATM knock-down resulted in multipolar spindle assembly or caused evident defects in chromosome alignment.

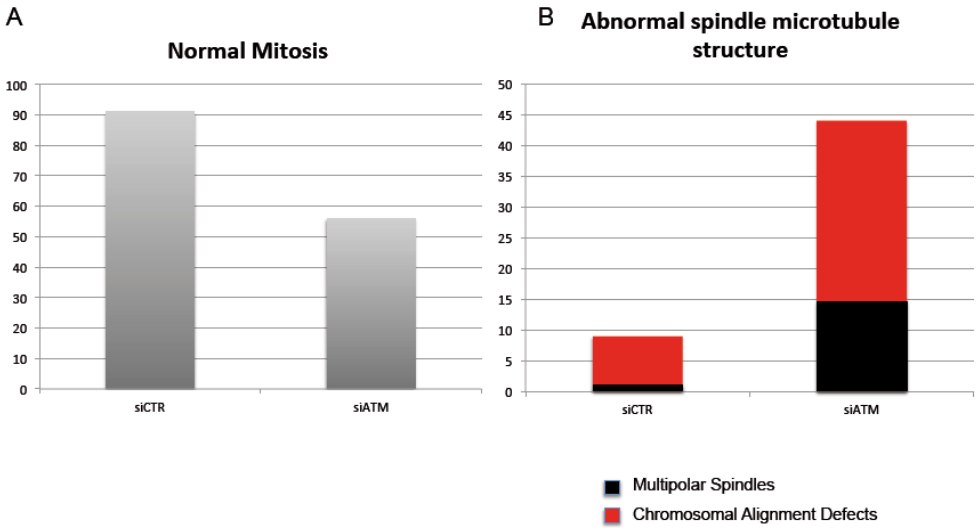

Fig 9. siRNA-induced ATM down-regulation increases the frequency of mitotic defects. A and B HeLa cells were synchronized in G2/M with 9 μM RO-3306 and then treated with DMSO or 10 μM Ku-55933 in fresh medium containing 40 μM MG-132.

ATM-depleted cells often contained extra spindle poles. In order to probe the structure of these poles, we stained Ku-55933-treated and ATM-silenced cells, and the respective controls, for the spindle pole protein NuMA1 and for the microtubule-nucleating protein □-tubulin, which is localized at centrosomes. Spindles in DMSO and in control siRNA-treated cells were in 90% of the cases bipolar, with one focus for □-tubulin at each pole and both poles being positive for NuMA1. 90% of multipolar spindles found in control cells showed multiple centrosomes, only 10% of the multipolar spindles had poles that were lacking the centrosome marker □-tubulin (Fig. 10-11-12). By contrast, ATM down-regulation by Ku-55933 or ATM siRNA resulted in five-fold increase of multipolar spindles in which the extra poles were positive for NuMA1 staining but lacked the centrosome marker □-tubulin. These data indicate that ATM down-regulation also favours centrosome-independent spindle pole assembly.

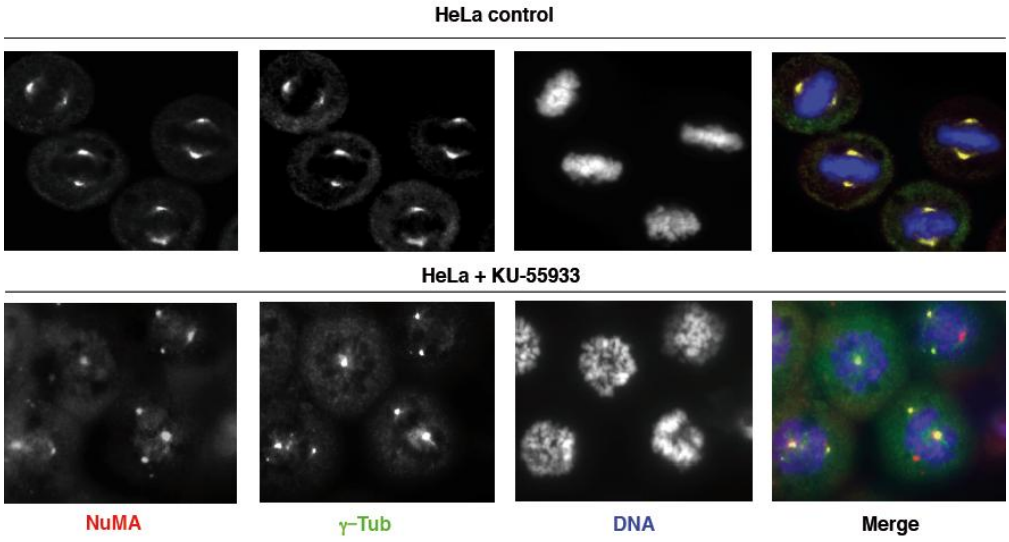

Fig 10. ATM chemical inhibition results in centrosome-independent spindle-pole assembly. HeLa cells grown on cover-slide were synchronized in G2/M with 9 μM RO-3306. After 20 hours, cells were released in the fresh medium containing 40 μM MG-132 in presence of DMSO or 10 μM Ku-55933. After 2 hours, cells were stained for the centrosome marker γ–tubulin, for the spindle-pole protein NuMA1 and the DNA. In the merge, NuMA staining is shown in red, γ–tubulin in green and the DNA in blue. ATM inhibition resulted in assembly of exogenous spindle poles, positive for the NuMA1 staining but negative for the centrosomal protein γ–tubulin.

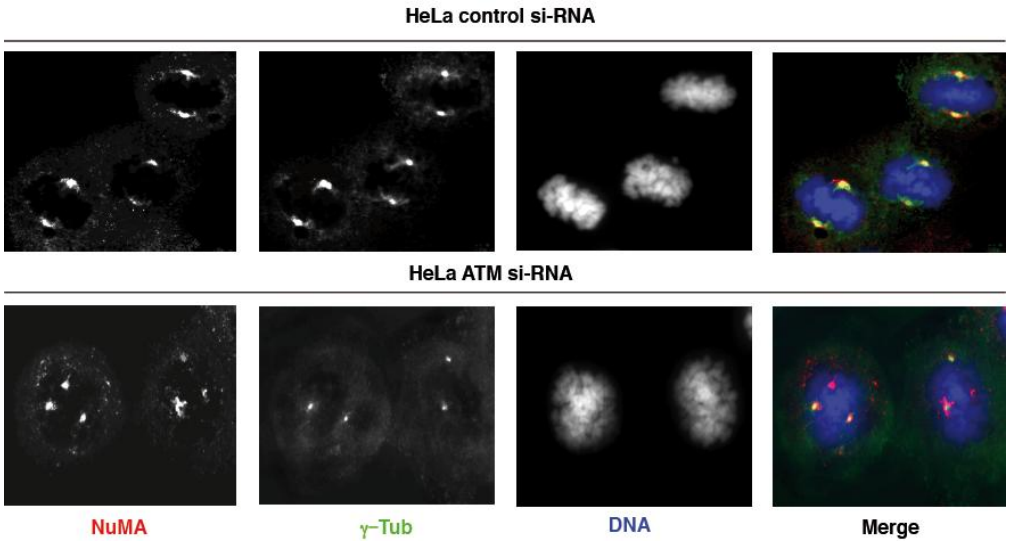

Fig 11. ATM knock-down results in centrosome-independent spindle pole assembly. Control siRNA and ATM siRNA HeLa cells grown on cover-slide were synchronized in 9 μM RO-3306. After RO-3306 wash out, cells were arrested for 2-hours in metaphase with 40 μM MG-132 and processed for IF. Staining for γ–tubulin (red), NuMA1 (green) and DNA (blue) showed that ATM knock-down by siRNA increased the frequency of γ–tubulin negative, NuMA1-positive multipolar spindles.

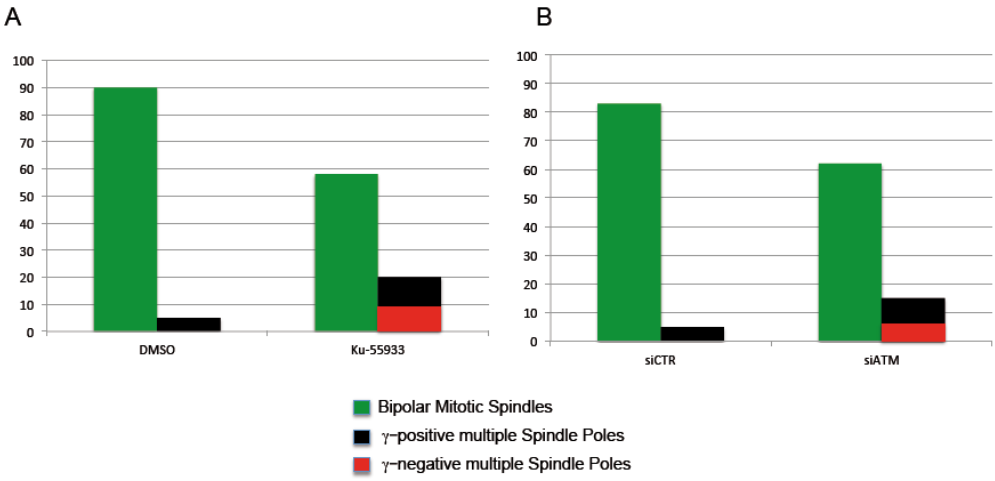

Fig. 12. ATM knock-down increases the frequency of γ–tubulin-negative extra-poles. DMSO or Ku 55933-treated (A) and control or ATM siRNA (B) HeLa cells were synchronized in 9 μM RO-3306. After RO-3306 wash out, cells were arrested for 2-hours in metaphase with 40 μM MG-132 and stained for γ-tubulin and NuMA1. 60-80 cells for each cell population were scored. In control cells, 90% of the spindles were bipolar; only 10% were multipolar. Among the multipolar 95% were positive for γ-tubulin, suggesting centrosome amplification or splitting. By contrast the Ku-55933-treated cells and the ATM knocked down cells showed not only an increase in γ–tubulin-positive multiple spindle poles but also a dramatic increase in the percentage of NuMA1-positive, γ-tubulin-negative ones.

Nevertheless, upon longer incubation in MG-132, most of the ATM down-regulated cells finally reached a bipolar configuration. Indeed, we observed that the □□tubulin negative-NuMA1 positive poles clustered with the □□tubulin positive-NuMA1 positive poles forming a pseudo-bipolar spindle. The clustering of multipolar spindles into pseudo-bipolar spindles has been shown to increase the frequency in merotelic attachment and ultimately led to an increase in lagging chromosome in anaphase (Ganem et al, 2009). Indeed, we found that anaphases in Ku-55933- and ATM siRNA-treated cells showed higher frequency of lagging chromosomes compared to the control cells, 26% vs. 5%, respectively. (Fig13).

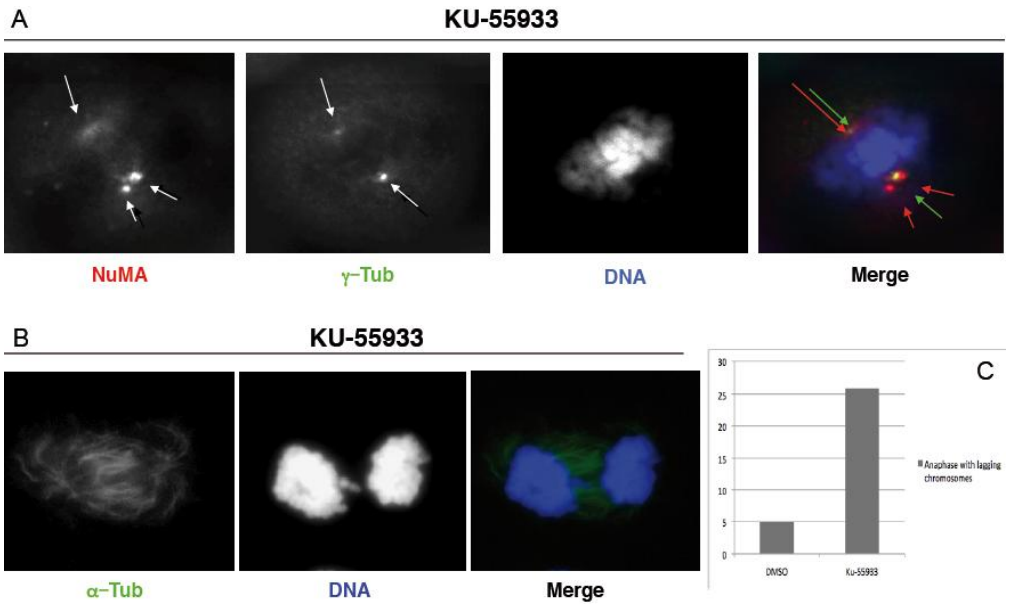

Fig. 13. ATM down-regulation induces assembly of pseudo-bipolar spindles by acentrosomal spindle-pole coalescence and increased frequency of lagging chromosomes in anaphase. A HeLa cells were synchronized with 9 μM RO-3309. After 20-hours of treatment, cells were released from RO-3306 and incubated with MG-132 in presence or absence of 10 μM Ku-55933 for 4-hours. Cells were stained for NuMA1, γ-tubulin and DNA. In the merge, NuMA1 is shown in red, γ-tubulin in green and the DNA in blue. **B** Cells were treated as in A and stained for α-tubulin and DNA. In the merge, α-tubulin is shown in green and the DNA in blue. **C**, Quantification of anaphases characterized by lagging chromosome visualized in 60-80 cells treated and stained as in B.

We performed same experiment in the non-transformed, chromosomally-stable, human cell line derived from retinal pigment epithelial cells immortalized with telomerase (hTERT-RPE1). Cells were grown on cover-slide and treated, after RO-3306 synchronization, with or without Ku-55933 in presence of MG-132. Ku-55933 treatment of hTERT-RPE cells led to a 15% of chromosomal alignment defects and to a 50% of spindles that were displaced from the cell centre (in 93% of deranged mitosis), indicating defects in the regulation of astral-microtubules attachment to the cell cortex, a fundamental interaction in the control of spindle orientation (Fig. 14; Radulescu et al. 2010).

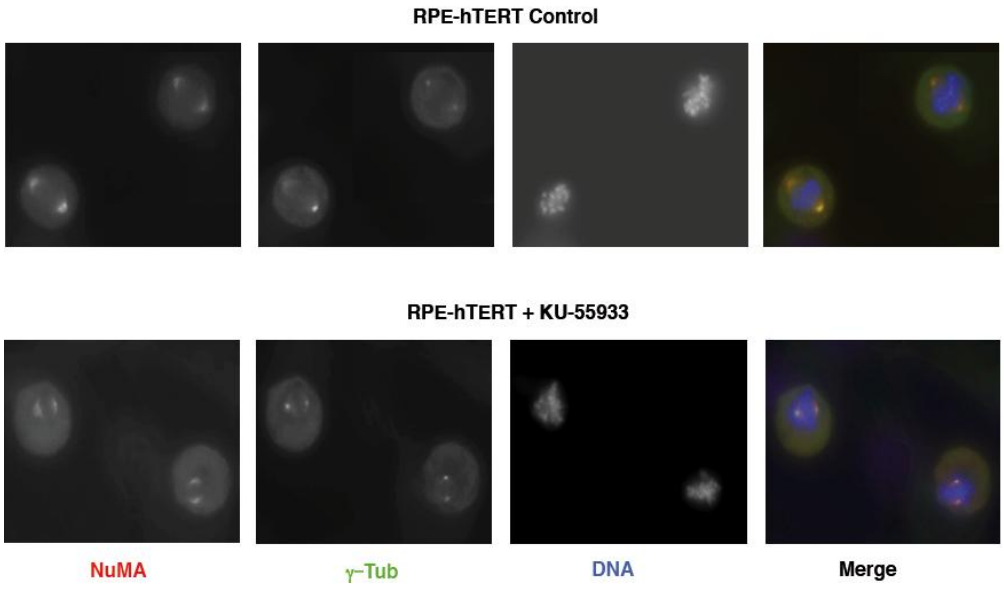

Fig 14. ATM down-regulation results in defective chromosome alignment and in displacement from the cell center in RPE-hTERT cell line. RPE-hTERT cells were plated on cover-slide 24-hours before RO-3306 treatment. After 20 –hours treatment with 9 μM RO-3306, cells were released in a fresh medium containing 40 μM MG-132 in presence or in absence of 10 μM Ku-55933. After 2-hours treatment, cells were stained for NuMA1, γ-tubulin and the DNA and were analysed by IF. In the merge, NuMA1 staining is shown in red, γ-tubulin in green and the DNA in blue). ATM down-regulation caused defective chromosome alignment on the metaphase plate and spindle displacement from the cell centre but not significant increase in multipolar spindles.

These data suggest that ATM may be involved in several processes that control spindle assembly: 1. regulation of spindle poles focusing at centrosomes; 2. maintenance of clustered extra-centrosomes at one pole in cells with extra centrosomes (Ganem et al, 2009); 3. interaction of astral microtubules with the plasma-membrane at the cell cortex. All these cellular processes have been described to involve the protein NuMA1 (Quintyne et al. 2005; Radulescu and Cleveland, 2010).

The phenotype obtained by ATM expression silencing and by ATM kinase chemical inhibition resembled the phenotypes described by Tankyrase-1 gene silencing (Chang et al., 2005). Tankyrase-1 is a poly(ADP-ribose) polymerase (PARP), also called PARP-5, able to use NAD^+ as substrate to generate ADP-ribose polymers onto glutamic acid residues of protein acceptors. The result is a post-translational modification that can drastically alter the properties of the acceptor proteins. Indeed, Tankyrase-1 has been shown to be able to interact with and poly(ADP-ribose)ylate NuMA1, and also TRF1 and IRAP (Insulin-Responsive Aminopeptidase) (Sbodio and Chi, 2002). In addition, Tankyrase-1 co-immunoprecipitates with NuMA1 (Nuclear Mitotic Apparatus Protein-1) and regulates the PARylation of major mitotically PARylated proteins (Chang W. et al., 2005). NuMA1 is a large nuclear protein component of the nuclear

matrix in interphase, that moves to the spindle pole, after the nuclear envelope breaks down, in mitosis via its ability to interact with dynein and microtubules. In mitosis, NuMA1 is responsible for tethering microtubule minus-ends at the spindle pole via its microtubule-binding sites. NuMA1 has also a central role in asymmetric cell division, a relevant mechanism to the determination of cell fate during development and to the specification of stem cell self-renewal versus differentiation. Indeed, a subset of NuMA1 molecules is preferentially recruited to one part of the cell cortex where it mediates spindle anchoring via the associated protein LGN and G□□□Lechler and Fuchs, 2005; Radulescu and Cleveland, 2010). Moreover, it has been showed that NuMA1 hyper-expression, observed in many tumour cells, led to abnormal mitosis and centrosome amplification. A role for NuMA1 has also been suggested in the clustering of extra-centrosome to form pseudo-bipolar spindles (Quintyne et al. 2005).

In order to meet its mitotic functions, NuMA1 has to be PARylated in a tankyrase-1-dependent manner. Indeed, tankyrase-1 gene silencing resulted in pre-anaphase arrest, chromosome misalignment, centrosome-independent spindle pole assembly and displacement of the spindle from the cell centre (Chang P. et al. 2005).

In order to determine whether the phenotype caused by ATM down-regulation were due to impaired poly(ADP-rybosil)ation of NuMA1, PARylated proteins were immunoprecipitated from nocodazole arrested HeLa cells that were further incubated for 1-hour treatment in the absence or presence of the ATM inhibitor Ku-55933 (Fig. 15A). Western blot showed that NuMA1 co-immunoprecipitated with PAR proteins in nocodazole arrested cells, however, ATM kinase inhibition dramatically reduced the NuMA1 PARylation. Moreover, Tankyrase-1 was auto-PARylated in nocodazole-treated HeLa cells while ATM inhibition led to a reduced gel mobility of Tankyrase-1 and a small reduction in PARylated Tankyrase-1. NuMA1 PARylation was also drastically reduced in ATM interfered HeLa cells with respect to the control interfered cells (Fig15C). Tankyrase-1 auto-PARylation was also only slightly reduced ATM siRNA transfected cells (Fig15C).

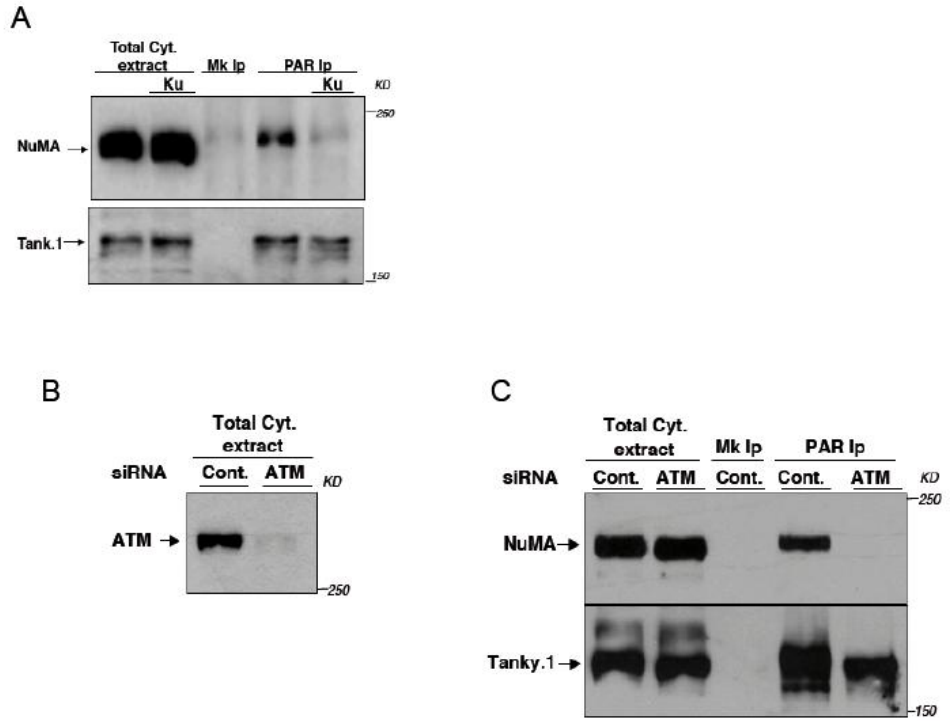

Fig 15. ATM down-regulation results a dramatic reduction of NuMA1 PARylation. A Nocodazole arrested HeLa cells were treated or not for 1-hour with 10 μ□□Ku-55933. Cells were harvested and PAR proteins were immunoprecipitated. NuMA1 and Tankyrase-1 were detected by western blot. **B** The efficiency of ATM knock-down. Control siRNA and ATM siRNA transfected HeLa cells were arrested in Nocodazole and harvested at day 3 after transfection. Western blot for ATM was performed **C** Mock-transfected cells and ATM depleted cells were synchronized with Nocodazole. At day 3 after transfection, cells were harvested and lysed. PAR proteins were immunopreciptated. Total extracts and Immunoprecipitation's samples were analysed by western blot for NuMA1 and Tankyrase-1

To understand how ATM could affect the PARylation of NuMA1, we asked whether ATM and Tankyrase-1 physically interacted with each other. We probed ATM immunoprecipitates with an anti-tankyrase-1 antibody and viceversa from asynchronous and nocodazole-arrested cells. Immunoblots demonstrated that the Tankyrase-1 signal was readily detectable in ATM immunoprecipitates and viceversa both in interphase and in mitosis (Fig 16A). The interaction was also confirmed in HCT-116 and in RPE-hTERT (Fig16 B, C, D).

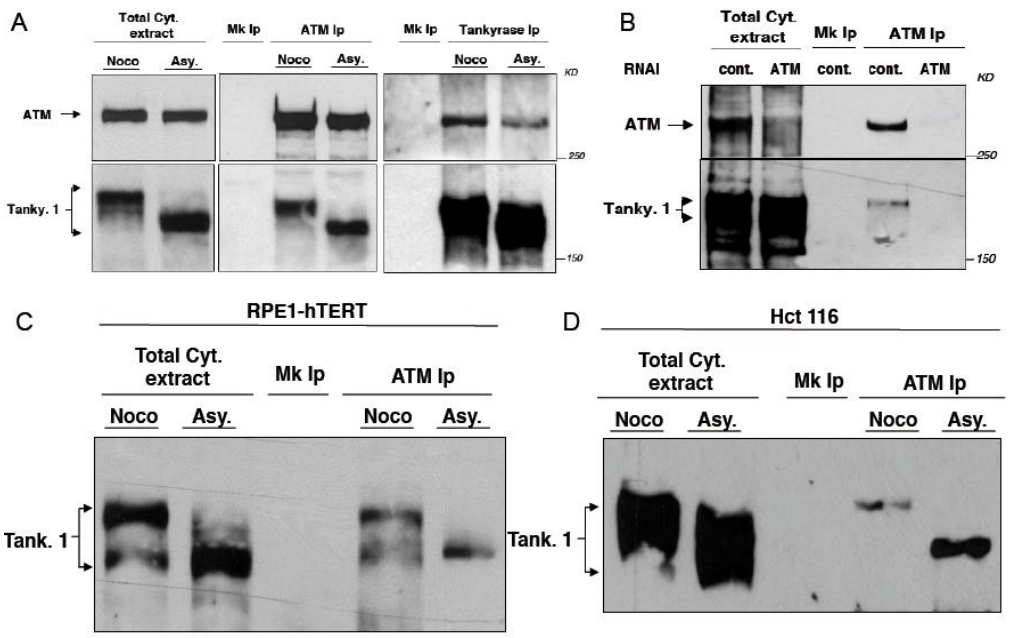

Fig 16. ATM interacts with Tankyrase-1 in different human cell lines. A ATM and Tankyrase-1 were immunoprecipitated from Nocodazole arrested and from asynchronous HeLa cells. **B** At day 3 after transfection, ATM was immunoprecipitated from control cells and from ATM-depleted cells. Western blot for ATM and Tankyrase-1 confirmed the specificity of the antibody. **C and D** ATM was immunoprecipitated from Nocodazole arrested and from asynchronous RPE-hTERT (**C**) and from HCT-116 cells (**D**). Western blot for Tankyrase-1 confirmed the interaction between ATM and Tankyrase-1.

Both ATM and Tankyrase-1 have been described to interact with TRF1 in human cells (Kishi et al. 2001; Sbodio and Chi, 2002). By contrast mouse TRF1 lacks the Tankyrase-1 binding domain and is not able to interact with Tankyrase-1 (Sbodio and Chi, 2002). To investigate if the interaction between ATM and tankyrase-1 was mediated by TRF, ATM was immunoprecipitated from mouse NIH-3T3 cells, western blot demonstrated that the interaction between ATM and Tankyrase-1 was conserved in mouse, demonstrating that the interaction was TRF1-independent (Fig17).

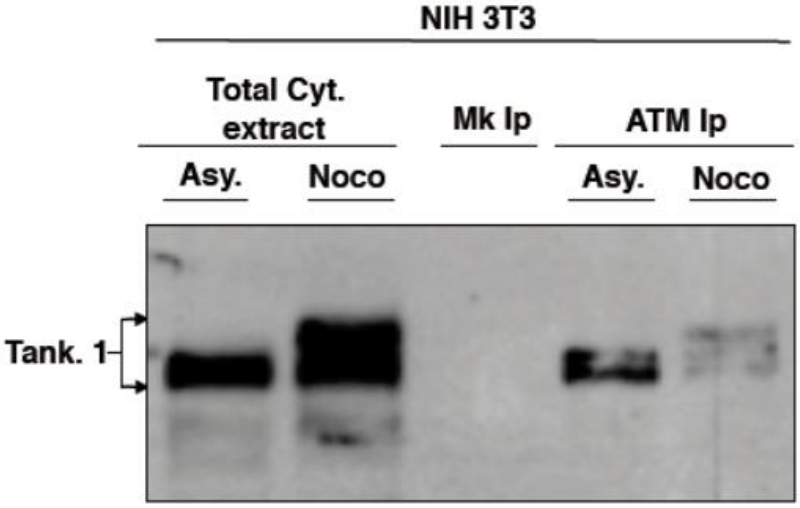

Fig 17. The interaction between ATM and Tankyrase-1 is TRF1-independent. ATM was immunoprecipitated from asynchronous and from Nocodazole arrested NIH3T3 cells. ATM was immunoprecipitated and then fractionated by SDS-PAGE. Tankyrase-1 detection by western-blot confirmed the interaction between ATM and Tankyrase-1 in mouse and indicated that the interaction was TRF1-independent.

To better analyze the physical interaction between ATM and tankyrase-1, tankyrase-1 deletion constructs, fused to the 3XFlag epitope, were generated and transfected in HeLa cells. The first construct encodes a Tankyrase-1 version (3F-T1) that lacks the N-terminal HPS (Homopolymeric region domain reach in Histidine, Proline and Serine), the second encodes a tankyrase-1 version (3F-T2) that lacks the C-terminal SAM domain and PARP catalytic domain; both 3F-T1 and 3F-T2 conserved the Ankyrin domain. 3F-T1 and 3F-T2 expressed differentially in transfected HeLa cells (Fig18A). Co-immunoprecipitation experiments demonstrated that ATM was able to interact with both 3F-T1 and 3F-T2 according to the different genes expression levels (Fig. 18 B, C). Since the common part of the two versions is the central repetitions of ankyrin domain, we conclude that ATM interacts with tankyrase-1 via the tankyrase-1 ankyrin domains.

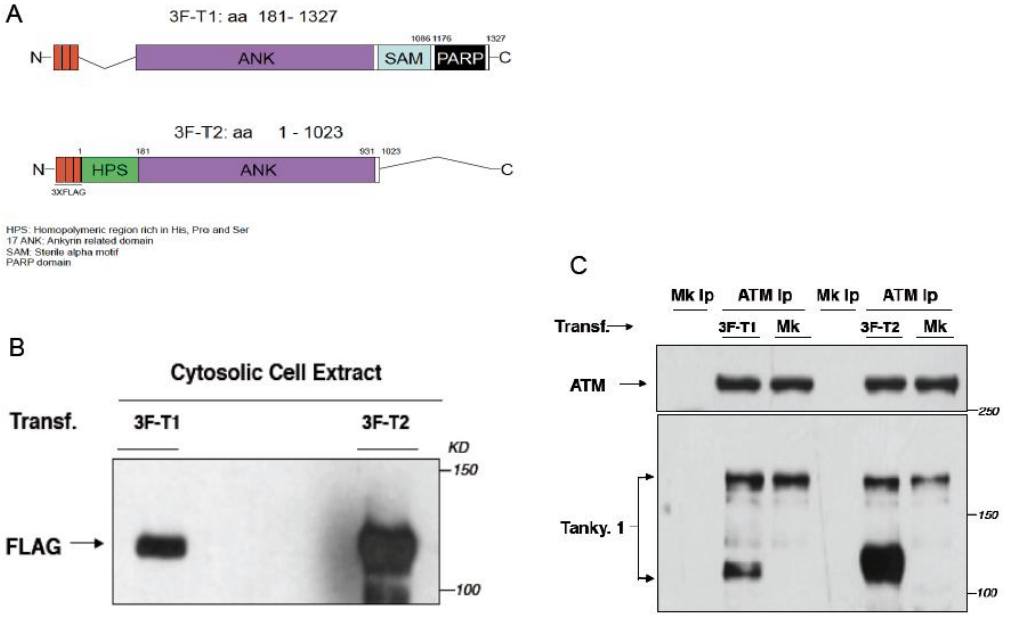

Fig 18. The Tankyrase-1 ANK domain is essential for the interaction between ATM and Tankyrase-1. **A** two constructs of Tankyrase-1 were generated, both fused with FLAG. **B** Western blot for FLAG showed that the two constructs expressed differently in HeLa cells. **C** ATM was immunoprecipitated from 3F-T1, from 3F-T2 and from mock transfected HeLa cells. Tankyrase-1 western blot demonstrated that ATM interacted with endogenous Tankyrase-1 and both exogenous Tankyrase-1, according with expression level.

We asked whether ATM inhibition affected Tankyrase-1-dependent PARylation of NuMA1 by interfering with the ATM-tankyrase complex formation. However, ATM chemical inhibition did not appear to impair the binding between ATM and Tankyrase-1 (Fig. 19A). Moreover, we found that ATM was able also to interact with NuMA1 (Fig. 19A).

NuMA1 was, nevertheless, dispensable for the interaction between ATM and Tankyrase-1 (Fig. 20B). Indeed ATM-tankyrase-1 binding was detected both in interphase and in mitosis, while NuMA1, a tightly nuclear protein during interphase that becomes cytosolic soluble after nuclear envelope breakdown, was not well recovered in our cytosolic lysates from interphase cells (Fig. 19B).

Nevertheless, we found that NuMA was phosphorylated at S(T)/Q sites in nocodazole-arrested cells while, moreover, ATM down-regulation led to a significant reduction in NuMA1 phosphorylation at S(T)/Q sites. Thus, NuMA1 was phosphorylated in mitosis in ATM-dependent manner (Fig. 19C). Our findings may suggest that tankyrase-dependent NuMA PARylation may require ATM-dependent phosphorylation of NuMA.

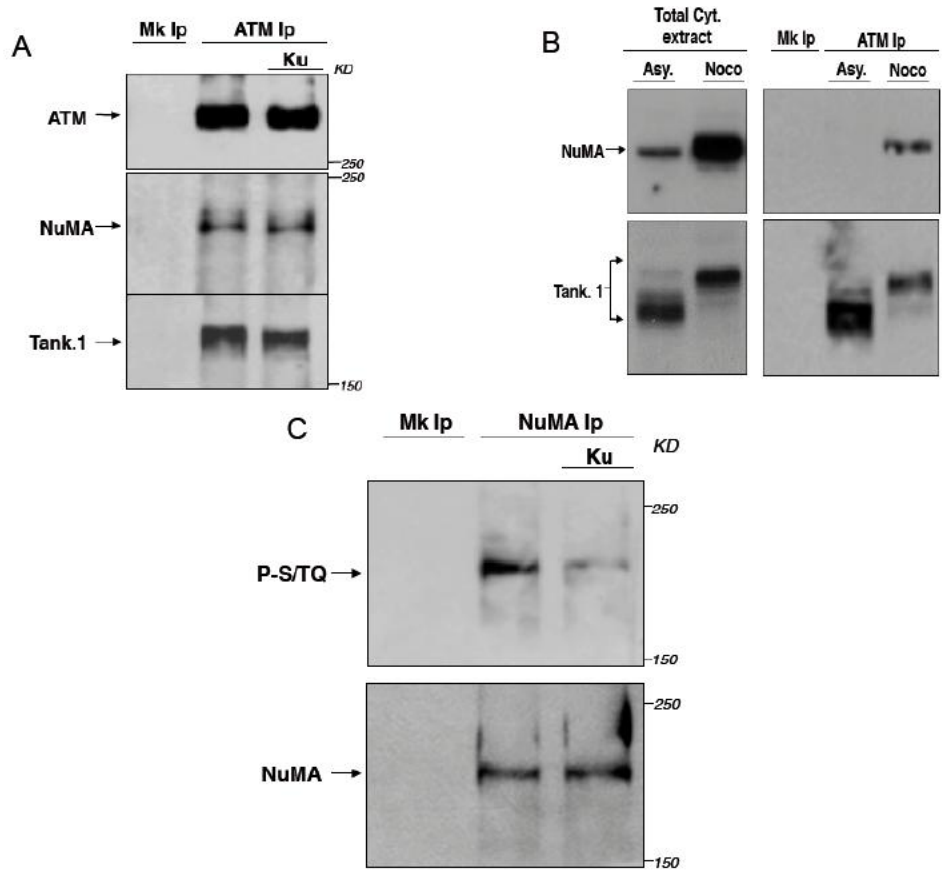

Fig 19. ATM kinase is not required for complex maintenance but it is required for NuMA1 phosphorylation. A ATM was immunoprecipitated from Nocodazole arrested cells treated 1-hour with or without 10 μM Ku-55933. Western blots for Tankyrase-1, NuMA1 and ATM were performed. **B** ATM-immunoprecipitation was performed from Asynchronous and Nocodazole arrested HeLa cells. Western blot for NuMA1 and Tankyrase-1 showed the mitotic interaction between NuMA, Tankyrase-1 and ATM **C** NuMA1 was immunoprecipitated from Nocodazole arrested HeLa cells, treated or not 1-hour with Ku-55933. Western blot for P-ATM/ATR substrates showed that NuMA was phosphorylated in mitosis in ATM-dependent manner.

Finally, our findings were confirmed by experiments performed in lymphoid cells derived from an A-T patient (GM03189 cell line). PAR proteins were immunoprecipitated from mitotic GM03189 lymphoid cells and control AHH1 lymphoid cells. While NuMA1 was readily detectable in PARylated protein immunoprecipitates from control mitotic cells, it could not be detected in immunoprecipitates from mitotic A-T GM03189 cells (Fig. 20). These data demonstrate that Tankyrase-1 dependent Poly(ADP-ribosyl)ation of NuMA1 protein is significantly depressed in cells of A-T patients.

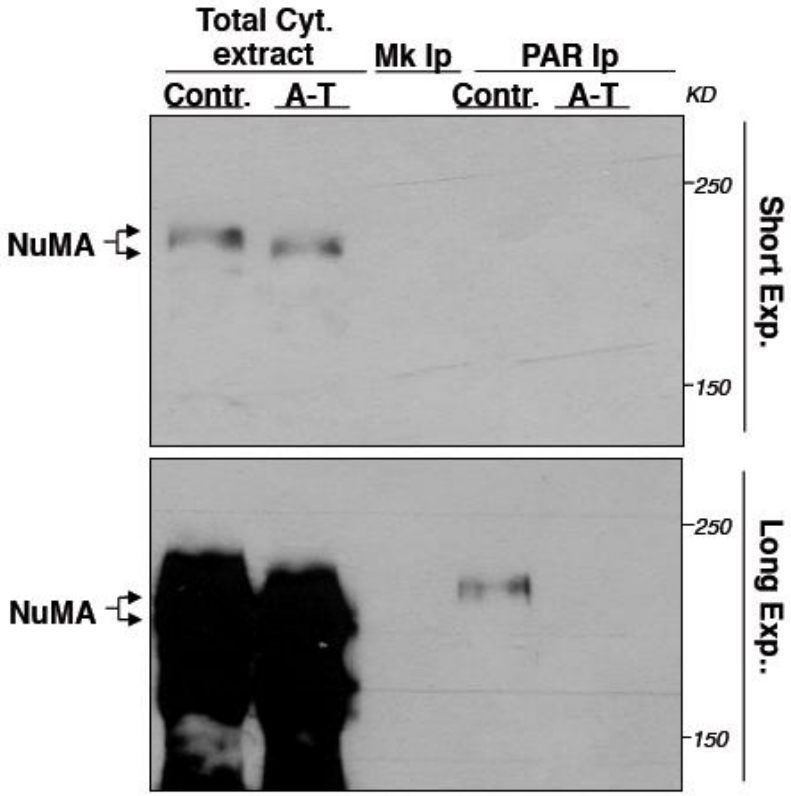

Fig 20. NuMA1 is not PARylated in lymphoblastic A-T cells. Control AHH1 and GM03189 A-T cells were first synchronized for 20-hours with 3 mM thymidine and then treated with 80 ng/mL Nocodazole for 11-hours. After Nocodazole treatment, cells were harvested and IP PAR was performed from lysates obtained from mitotic AHH1 and GM03189. Short and Long exposures of western blot for NuMA demonstrated that NuMA was not PARylated in GM03189 A-T cells.

3.5 **CONCLUSIONS**

Collectively, our data support a model in which ATM is not only required for DDR but also it is involved in other cellular processes impaired in A-T cells. We focused our studies on the causes of whole chromosome aneuploidy found in A-T cells and tissues (Shen et al. 2005; Iourov et al. 2009; Li et al. 2010). We found that ATM inhibition by chemical inhibition and siRNA led to the delayed and abnormal mitosis in human cancer cell lines and in human non-tumoral cell lines. Indeed, we found that ATM down-regulation resulted in increased defects in chromosome alignment, multipolar spindles and spindle displacement form cells center. The increase in multipolar spindles was found principally in tumor cells. In particular, ATM down-regulation led to an increase in centrosome-independent spindle pole assembly. Moreover, splitting of clustered-centrosomes was also found. In non-tumoral cells, ATM down-regulation resulted in defects in chromosome alignment and, principally, spindle displacement form cell center. This data suggest that ATM may be also involved in regulation of spindle orientation. All features obtained by ATM down-regulation resembled the phenotype obtained by Tankyrase-1 gene knock-down (Chang P. et al. 2005). Indeed, we found that ATM interacted with the PARP Tankyrase-1 in TRF1-indepenent manner and that ATM down-regulation impaired significantly the Poly(ADP-rybosil)ation of NuMA1, the principal mitotic substrate of Tankyrase-1. We showed that ATM interacted with Tankyrase-1 during every phase of cell-cycle; however, during mitosis ATM interacted also with NuMA1 and regulated the Tankyrase-1-dependent PARylation, perhaps through ATM-dependent phosphorylation of NuMa1.

Tankyrase-1 has been shown to be required for insulin-stimulated translocation of the glucore transporter Glut-4 into the plasmamembrane (Yeh et al., 2007). It has also been shown that tankyrase-1 interacts with the Telomere Repeat binding Factor-1 (TRF1) and the Tankyrase-1-dependent poly(ADP-ribosyl)ation (PARylation) of TRF1 is required to promote Shelterin complex disassembly to allow telomerase-dependent telomere elongation in human cells (Smith S. and de Lange, 2000).

Our data suggest that ATM may be involved in key cellular processes like regulation of mitotic spindle assembly, telomere-length regulation and insulin-response via its interaction with tankyrase-1 and regulation of tankyrase-1-dependent protein PARylation. These findings provide a mechanistic explanation for several A-T syndrome features. ATM is also a gene mutated in many human cancers, and *Atm* heterozygous carries show an increased susceptibility to develop breast cancer (Walsh and King 2007; Campeau et al. 2008). We believe that our findings may help to uncover new biochemical markers to identify *Atm* carriers with increased cancer susceptibility and shed light on new therapeutic approaches for the A-T syndrome.

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